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COLORIMETRIC METHOD FOR DETERMINING IRON IN BLOOD

George Evans, Hopatcong, and Ronald Searcy, Upper Montclair, N.J., assignors to Hoffmann-La Roche Inc., Nutley, N.J., a corporation of New Jersey
No Drawing. Filed Dec. 19, 1967, Ser. No. 691,703
Int. Cl. G01n 31/22

U.S. Cl. 23—230

14 Claims

ABSTRACT OF THE DISCLOSURE

A colorimetric method for quantitatively determining the iron content of whole blood and serum by extracting the iron from the whole blood or serum in a water soluble ferrous state and treating the ferrous ions with 5-(2-pyridyl)-2H-1,4-benzodiazepines and water soluble salts thereof to produce a brilliant purple colored solution which can be quantitated by standard colorimetric means.

BACKGROUND OF THE INVENTION

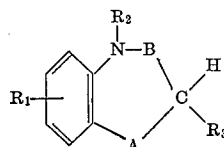
Techniques have been developed for quantitatively determining the iron content of whole blood and serum by means of reacting the iron in either its ferric or ferrous state with various color forming reagents such as ammonium thiocyanate, bathophenanthroline, dipyriddy, etc. However, this process has suffered from several disadvantages due to the fact that known color forming reagents are too sensitive to extraneous sources so that they pick up trace contaminants. In many cases these color forming reagents do not produce a sufficient color differentiation between the blank and the sample prepared from whole blood or serum to be tested. This makes the quantitative determination of the iron content in this sample difficult to carry out by standard colorimetric instruments such as a Beckman Spectrophotometer.

The colorimetric technique for quantitating iron has been important in determining the hemoglobin content of whole blood since by utilizing a standard conversion factor the hemoglobin present in whole blood can be calculated from the iron content of the blood sample. However, the liberation of the iron from the hemoglobin to place it in a water soluble state and in a state where it can be quantitated colorimetrically has proven too complicated and time-consuming for routine diagnostic use. This procedure of liberation and solubilization of the iron from the hemoglobin involves releasing iron in a water soluble state by first adding concentrated sulfuric acid and potassium persulfate at room temperature, and thereafter removing the protein by precipitation by the addition of sodium tungstate.

The procedure for liberating iron from serum in a water soluble state, where the quantity of iron can be determined by use of a color forming reagent, has also proven to be too tedious and complicated for routine diagnostic use since the iron was liberated from the serum by ashing.

SUMMARY OF THE INVENTION

In accordance with this invention we have discovered that when a compound selected from the group consisting of compounds of the formula:



(I)

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wherein A is selected from the group consisting of



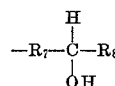
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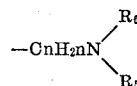
B is selected from



and $\text{—CH}_2\text{—}$; R_1 is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R_2 is selected from the group consisting of



hydrogen, lower alkyl and



n is an integer from 2 to 7; R_3 is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; R_4 is 2-pyridyl; R_5 is selected from the group consisting of lower alkyl and hydrogen; R_6 is selected from the group consisting of lower alkyl, hydrogen,



and R_5 and R_6 where taken together with their attached nitrogen atom form a radical selected from the group consisting of piperazinyl, lower alkyl substituted piperazinyl, pyrrolidinyl, lower alkyl substituted pyrrolidinyl, piperidinyl and lower alkyl substituted piperidinyl; R_7 is lower alkyl; and R_8 is selected from the group consisting of lower alkyl and hydrogen

and water soluble salts thereof are added to an aqueous solution containing ferrous ions prepared from whole blood or serum, a purple colored solution is obtained which can be quantitated as to its iron content by standard colorimetric means.

The color differentiation with different concentrations of ferrous ions produced by the compound of Formula I above, is such that the concentration of iron therein can be easily determined by standard colorimetric instruments. Furthermore, the compound of Formula I is not sensitive to extraneous sources so as to include interferences from trace contaminants of iron. Therefore, the method of this invention provides a simple colorimetric means of quantitatively determining the iron content of blood serum and whole blood.

By means of utilizing the compound of Formula I above, as a color forming reagent, the iron content of hemoglobin can be quantitatively determined by colorimetric means without the necessity of first treating the whole blood sample with a mixture of sulfuric acid and potassium persulfate and then removing the protein by the addition of sodium tungstate. In accordance with this invention, the hemoglobin in a whole blood sample can be determined by first extracting the iron into an aqueous solution in the ferric state from the hemoglobin by addition of sodium hypochlorite, thereafter reducing the ferric ions to ferrous ions, reacting the ferrous ions with the compound of Formula I above to produce a brilliant deep color and colorimetrically quantitating the iron present in the solution. The quantity of hemoglobin present therein can be calculated utilizing a

standard conversion factor based upon the quantity of iron present in the hemoglobin molecule. As can be seen, the above procedure provides a simple and quick method for quantitatively determining the hemoglobin content of whole blood, which is ideally suited for routine diagnostic use.

In the case of blood serum, the iron content can be determined by use of the compound of Formula I without the necessity of ashing in order to extract the iron from serum into an aqueous solution. In accordance with this invention, the iron is first liberated from the serum in an aqueous solution in the ferric state by extraction with an acid deproteinizing agent. The ferric ion is then reduced to the ferrous state. The ferrous ion in the aqueous solution is then reacted with the compound of Formula I above to produce a brilliant deep purple color. The iron content in the resulting purple solution can be quantitatively determined by standard colorimetric means.

DETAILED DESCRIPTION OF THE INVENTION

The term "lower alkyl" as used throughout this specification includes both straight and branched chain alkyl groups having from 1 to 7 carbon atoms such as methyl, ethyl, propyl, isopropyl and the like. The term "lower alkanoyloxy" refers to both straight chain and branched chain aliphatic carboxylic acid moieties, such as acetoxy, propionyloxy, butyryloxy and the like. The term "halogen" includes bromine, chlorine, fluorine and iodine. Also included within the purview of the present invention are the water soluble acid addition salts of the compounds of Formula I above. Any conventional water soluble acid addition salts of the compounds of Formula I above, may be utilized in the process of this invention to quantitatively determine the iron content of aqueous solutions. Among the acid addition salts which can be utilized in accordance with this invention, includes salts of compounds of the Formula I with organic or inorganic acids such as hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, acetic acid, formic acid, succinic acid, maleic acid, p-toluene sulfonic acid and the like.

Examples of benzodiazepine compounds of Formula I above which are particularly suitable as the color forming reagent in the process of this invention include the following:

7-bromo-1,3-dihydro-1-[4-(4-methyl-1-piperazinyl)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
7-amino-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
1-methyl-1-[3-(7-bromo-5-(2-pyridyl)-1,3-dihydro-2-oxo-2H-1,4-benzodiazepin-1-yl)propyl]urea

whose preparation is disclosed in U.S. patent application Ser. No. 677,092, filed Oct. 23, 1967 in the name of Earley et al.;

7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepine;
7-amino-1,3-dihydro-1-methyl-5-(2-pyridyl)-1H-1,4-benzodiazepine;
7-bromo-1,3-dihydro-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one 4-oxide;
7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
7-bromo-1,3-dihydro-1-(β -hydroxypropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one; and
7-bromo-5-(2-pyridyl)-1,3-dihydro-1-[3-(N-cyanomethylamino)propyl]-2H-1,4-benzodiazepin-2-one

whose preparation is disclosed in U.S. patent application Ser. No. 677,092, filed Oct. 23, 1967 in the name of Earley et al.

By utilizing the color forming reagent of Formula I above, one can quantitate the iron content of serum

without the necessity of utilizing ashing to liberate or extract the iron from the serum. In accordance with this invention, iron can be liberated from serum by means of treating the serum with an acidic deproteinizing agent. The acidic deproteinizing agent extracts the iron from the serum in the ferric state. In extracting iron from the blood serum, any conventional acidic deproteinizing agent such as trichloroacetic acid, nitric acid or perchloric acid can be utilized.

The aqueous solution which contains the free iron in the ferric state is then treated with a chemical reducing agent to convert the iron to the desired ferrous state. In general any known chemical reducing agent can be used in this step of the procedure. These include, for example, ascorbic acid, hydrazine sulfate, thioglycolic acid, sodium thioglycolate, sodium hydrosulfite, sodium metabisulfite, sodium bisulfite, sodium sulfite, hydroxylamine hydrochloride, hydroxylamine sulfate, hydroquinone, stannous chloride, etc. In the preferred embodiment of the invention, however, the chemical reduction of free iron from the ferric state to the ferrous state is carried out using thioglycolic acid or ascorbic acid as the reducing agent. The use of thioglycolic acid is advantageous since it permits the reduction of the free iron from the ferric state to the ferrous state to be accomplished at room temperature. The quantity of thioglycolic acid which is used in this step of the process is not particularly critical. A sufficient amount of the reducing agent should be used however to completely convert the free iron to the ferrous state. An excess of thioglycolic acid can be used, if desired, to insure complete conversion.

In a whole blood sample, the hemoglobin is determined by first liberating the iron from the fresh whole human blood in an aqueous solution in the ferric state. In general, the iron can be liberated from the sample by any convenient and conventional method. However, in accordance with this invention, the liberation of the iron can be accomplished by treating the blood at room temperature with a hypochlorite salt, preferably sodium hypochlorite. The use of a hypochlorite salt serves to liberate the iron in the form of ferric ions which when reduced to ferrous ions and then reacted with the compound of Formula I, will produce a color that can be quantitated so that the amount of iron in the blood can be determined. Any of the conventional methods of reducing the ferric ion liberated from the blood sample to the ferrous state such as hereinbefore described, can be utilized.

The quantity of hypochlorite salt which is used in the preferred embodiment of this invention is variable. However, a sufficient quantity of hypochlorite salt should be used to liberate all of the iron which is present in the blood. An excess of hypochlorite salt can be used, if desired, to insure that all of the iron present in this sample is liberated. The precise manner in which the hypochlorite salt is incorporated into the sample is not particularly critical. Generally, it will be introduced into the sample in the form of a dilute aqueous solution. In the preferred practice of this invention, the salt is added to the sample in the form of an aqueous solution containing from about 5 percent to about 25 percent by weight of a hypochlorite salt such as sodium hypochlorite.

When the iron has been liberated from the blood sample to be quantitated in the water soluble ferrous state, the compound of Formula I above is added to an aqueous solution containing the extracted ferrous ions. Upon mixing, a complexing takes place between the ferrous ion and the compound of Formula I above. The formation of the complex is evidenced by the development of a brilliant purple color. The precise manner in which the compound of Formula I above is added to the ferrous ion solution does not limit the scope or practice of this invention. Generally, however, the compound of Formula I above is added to the system in the form of a relatively dilute aqueous solution. In the preferred practice

of the invention, there is used an aqueous solution containing from about 0.25 percent to about 0.75 percent by weight of the compound of Formula I above.

The quantity of the compound of Formula I, which is added to the aqueous solution is variable. In all instances, however, a sufficient quantity of the compound of Formula I above, should be provided to react with all of the ferrous ions present in the aqueous solution. In order to insure, however, that all of the ferrous ions present in the mixture have been complexed, it is preferred to add a quantity of the color forming compound of Formula I above, which is in excess of that actually required to complex all of the ferrous ions which are available for complexing.

The desired purple coloration of the mixture will be noted immediately upon the addition of the compound of Formula I above, to the system. The color deepens as the reaction proceeds to completion. After standing for awhile at room temperature, color changes are no longer discernible to the naked eye. Accordingly, in order to insure uniform coloring, the aqueous solution should be allowed to stand until its color appears to have become constant. In general, it has been found that full development of the purple color will occur over a period of from about 5 to 20 minutes.

The quantitation of the iron in the colored sample can be carried out by any conventional colorimetric method utilizing standard spectrophotometers such as a Beckman Spectrophotometer, Coleman Junior spectrophotometer, etc.

The complex which is produced in the practice of the invention has its own spectral characteristics. Color was developed using a sample containing 6.0 μg . of iron. The optical density of the mixture was then measured at wave lengths ranging from 450–700 $m\mu$ in a Beckman Spectrophotometer, Model DBG, using a 1 cm. cuvette. With increasing wave length above 450 $m\mu$ there was a progressive rise in absorbance which reached a maximum at about 580 $m\mu$. The absorbance diminished at higher wave lengths.

In order to determine the speed and stability of the colorimeter response, a sample containing 6.0 μg . of iron was prepared. This sample was transferred to a 1 cm. cuvette immediately after the benzodiazepine salt color reagent had been added thereto. For a period of about one hour, optical density measurements were made continuously at 580 $m\mu$. It was found that the absorbance increased rapidly reaching a maximum value in about seven minutes and, further, that the measurement changed little thereafter.

The quantitative determination of iron in the sample is carried out as follows: the optical density of the purple colored sample is measured against a reagent blank at 580 $m\mu$ using a standard spectrophotometer, e.g. a Coleman Junior Spectrophotometer, employing a cuvette with 19 mm. light path. The quantity of iron in the specimen is determined in the conventional manner from the absorbance of the specimen with reference to a standard. The iron content in a sample such as serum is calculated according to the following formula:

Iron content of sample (mcg./100 ml.)

$$= \frac{\text{absorbance of Sample}}{\text{absorbance of Standard}}$$

$\times$ iron concentration of standard (mcg./100 ml.)

In determining the hemoglobin content of a whole blood sample, based upon the iron content thereof, the following formulation can be conveniently utilized

Hemoglobin in sample (grams/100 ml.)

= hemoglobin in standard (grams/100 ml.)

$$\times \frac{\text{absorbance sample}}{\text{absorbance standard}}$$

The hemoglobin in the standard is calculated by the following formula:

Hemoglobin in standard (grams/100 ml.)

$$= \frac{\text{Iron in standard (milligrams/100 ml.)}}{3.47}$$

The constant 3.47 is used for the conversion since it is now accepted that hemoglobin contains 0.347 percent of iron.

As indicated heretofore, the present invention provides an extremely important diagnostic and analytical tool. The described method can be used to determine the iron in various materials such as body fluids rapidly and accurately. More particularly, the method can be used to quantify the iron in blood serum or to determine the hemoglobin in fresh whole human blood based on its iron content. In addition to being a rapid and accurate method for making the determination, the results obtained by the test method are characterized by a high degree of reproducibility.

For a fuller understanding of the nature and objects of this invention, reference may be had to the following examples which are given merely as further illustrations of the invention and are not to be construed in a limiting sense.

Example 1

This example demonstrates the applicability of the test method to a blood hemoglobin determination.

In the method, a 20 μl . volume of whole blood was added to 2.0 ml. of an aqueous solution containing 17 percent by weight of sodium hypochlorite. To the mixture, thus obtained, was added 6.5 ml. of 2.7 percent thioglycolic acid prepared in 1 M sodium acetate. Subsequently, 1.5 ml. of an aqueous solution containing 0.33 percent of 7-bromo - 1,3 - dihydro - 1 - (3-dimethylaminopropyl) - 5-(2-pyridyl) - 2H - 1,4 - benzodiazepin-2-one dihydrochloride was added to the mixture. Almost immediately, a purple coloration was noted. The reaction mixture was allowed to stand for a period of about ten minutes to allow the color to fully develop. The optical density of the mixture was measured against a reagent blank at 580 $m\mu$ in a Coleman Junior Spectrophotometer using a cuvette with 19 mm. light path.

Using the data thus obtained, the hemoglobin content of the blood sample was calculated using the following formula:

Hemoglobin in sample (grams/100 ml.)

= hemoglobin in standard (grams/100 ml.)

$$\times \frac{\text{absorbance sample}}{\text{absorbance standard}}$$

The hemoglobin in the standard was calculated using the following formula:

Hemoglobin in standard (grams/100 ml.)

$$= \frac{\text{iron in standard (milligrams/100.)}}{3.47}$$

Test procedures the benzodiazepine, i.e., 7-bromo-1,3-dihydro - 1(3 - dimethylaminopropyl) - 5 - (2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride, described in the preceding paragraph were performed on blood samples obtained from healthy individuals and hospitalized patients. For comparative purposes, the hemoglobin content of the same blood samples was also determined by the cyanmethemoglobin method. In the latter procedure, a 20 μl . volume of whole blood was added to 5.0 ml. of an aqueous solution containing 1.0 gram per liter of sodium bicarbonate, 50.0 mg. per liter of potassium cyanide and 200 mg. per liter of potassium ferricyanide. After allowing a period of ten minutes for complete color formation, the optical density of each sample was measured against a reagent blank at 540 $m\mu$ in a Coleman Junior Spectrophotometer using a cuvette with a 19 mm. light

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path. The net absorbance was converted into hemoglobin concentrations by means of a standard curve constructed with dilutions of cyanmethemoglobin of known concentration.

The results obtained utilizing the aforesaid two techniques are set forth in the following table:

TABLE—DETERMINATION OF HEMOGLOBIN CONTENT
[Grams/100 ml]

Sample No.	Benzodiazepine test	Cyanmethemoglobin
1.....	15.5	15.2
2.....	10.9	11.0
3.....	14.2	13.9
4.....	10.0	10.3
5.....	14.8	15.2
6.....	15.2	15.2
7.....	13.3	13.4
8.....	4.6	4.8
9.....	14.4	14.3
10.....	5.8	5.6
11.....	13.0	13.0
12.....	16.0	16.2
13.....	11.3	11.6
14.....	12.0	12.0
15.....	15.1	15.5
16.....	15.8	15.8
17.....	13.2	13.2
18.....	14.4	14.4
19.....	14.4	14.4
20.....	12.0	12.0
21.....	10.4	10.2
22.....	12.5	12.2
23.....	12.2	12.1
24.....	11.8	11.4
25.....	11.2	10.9

Example 2

To a stirred solution of 19 g. (0.06 mole) of 7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one in 200 ml. of dry N,N-dimethylformamide was added portionwise 3 g. (0.063 mole) of 50% sodium hydride. The resultant solution was then cooled with an ice-water bath while 39 g. (0.18 mole) of 1,4-dibromobutane was added dropwise during 25 min. After stirring overnight at room temperature the solution was poured into 600 ml. of water and extracted with methylene chloride. The organic layer was separated, washed with brine, dried over sodium sulfate and concentrated. The resultant oil was dissolved in ether and then filtered from a small amount of precipitated solid (discarded). This filtrate was then put on silica gel and the silica gel washed with ether. The first 150-200 ml. of eluant was separated while the subsequent fractions (2 l. total) were concentrated to give 8 g. of a tan colored solid. The first ether fraction upon concentration gave an oil which was dissolved in a small amount of ether and seeded with a crystal from the 8 g. sample to give 7-bromo-1-(4-bromobutyl)-5-(2-pyridyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

EXAMPLE 3

A mixture of 9 g. (0.02 mole) of 7-bromo-1-(4-bromobutyl)-5-(2-pyridyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one, 3 g. (0.03 mole) of N-methylpiperazine, 3 g. (0.02 mole) of sodium iodide and 100 ml. of methylethyl ketone was stirred and refluxed for 22.5 hrs. Solvents were removed at reduced pressure and the residue partitioned between methylene chloride and water. The methylene chloride layer was washed with brine, dried over sodium sulfate and concentrated. Upon the addition of ether to the resultant oil a solid separated. The suspension was filtered and the solid discarded. The ether filtrate was concentrated and the residual oil was converted to the dihydrochloride by the addition of the theoretical amount of methanolic hydrogen chloride followed by ether. The resultant highly hygroscopic solid was recrystallized from ethanol-ether to give 7-bromo-1,3-dihydro[4-(4-methyl-1-piperazinyl)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride as white needles.

7-bromo-1,3-dihydro[4-(4-methyl-1-piperazinyl)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride was utilized in the same manner as 7-bromo-

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1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride to quantitate the iron in serum and the hemoglobin content of whole blood. The procedure utilized was the same as given in Example 1.

EXAMPLE 4

This example demonstrates the applicability of the test method to a serum iron determination.

The iron content in five different samples of serum was determined by utilizing the following procedure: 2 milliliters of clear unhemolyzed serum and 1.0 milliliters of 2 N hydrochloric acid was allowed to stand for 20 minutes. After standing, 3 milliliters of 17 percent by weight trichloro acetic acid aqueous solution was added, under constant stirring, to the serum. After adding the trichloro acetic acid, the serum was centrifuged at 2,500 r.p.m. for 15 minutes. 4 milliliters of the clear supernatant was treated with 2.0 milliliters of a 25 percent by weight ammonium acetate and 75 percent by weight water solution which contained 0.1 percent by weight of 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride. The optical density of the mixture was measured against a reagent blank at 580 m μ in a Coleman Junior Spectrophotometer using a cuvette with a 19 mm. light path. The iron content of the serum sample was calculated in the manner hereinbefore mentioned.

The iron content of these 5 samples was also determined by the method given by D. S. Fisher et al. utilizing tripyridyltriazine as described in Clin. Chem., 10: 21, (1964).

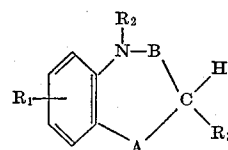
The results obtained utilizing the aforesaid two techniques are set forth in the following table:

DETERMINATION OF SERUM IRON CONTENT (μ g.)		
Sample No.	Benzodiazepine test (μ g./100 ml.)	Fisher et al.
1.....	83	78
2.....	107	111
3.....	95	99
4.....	75	79
5.....	110	118

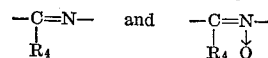
We claim:

1. A process for quantitating the iron content of whole blood comprising:

- extracting the iron in said blood into an aqueous solution in the ferric state;
- treating the ferric ions in said aqueous solution with a reducing agent to reduce the ferric ions to ferrous ions;
- reacting the ferrous ions with a compound selected from the group consisting of compounds of the formula



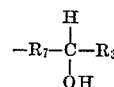
wherein A is selected from the group consisting of



B is selected from

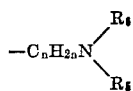


and -CH₂-; R₁ is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R₂ is selected from the group consisting of



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hydrogen, lower alkyl and



n is an integer from 2 to 7; R_3 is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; R_4 is 2-pyridyl; R_5 is selected from the group consisting of lower alkyl and hydrogen; R_6 is selected from the group consisting of lower alkyl, hydrogen,



and $-C\equiv N$; and R_5 and R_6 where taken together with their attached nitrogen atom form a radical selected from the group consisting of piperaziny, lower alkyl substituted piperaziny, pyrrolidiny, lower alkyl substituted pyrrolidiny, piperidiny and lower alkyl substituted piperidiny; R_7 is lower alkyl; and R_8 is selected from the group consisting of lower alkyl and hydrogen

and water soluble acid addition salts thereof to produce colored solutions; and

(d) colorimetrically quantitating the iron present in the solution by means of said color.

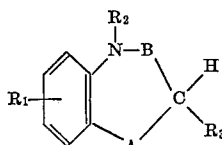
2. The process of claim 1 wherein the iron is extracted from the whole blood by the addition of sodium hypochlorite.

3. The process of claim 1 wherein said compound is 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

4. The process of claim 1 wherein said compound is 7-bromo-1,3-dihydro-1-[4-(4-methyl-1-piperaziny)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

5. A process of quantitating the iron content in serum comprising:

- extracting the iron from said serum into an aqueous solution in the ferric state;
- treating said solution with a reducing agent to reduce the ferric ions to the ferrous state;
- reacting the ferrous ions in said solution with a compound selected from the group consisting of compounds of the formula



wherein A is selected from the group consisting of



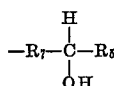
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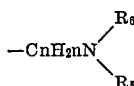
B is selected from



and $-CH_2-$; R_1 is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R_2 is selected from the group consisting of



hydrogen, lower alkyl and



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n is an integer from 2 to 7; R_3 is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; R_4 is 2-pyridyl; R_5 is selected from the group consisting of lower alkyl and hydrogen; R_6 is selected from the group consisting of lower alkyl, hydrogen,



and $-C\equiv N$; and R_5 and R_6 where taken together with their attached nitrogen atom form a radical selected from the group consisting of piperaziny, lower alkyl substituted piperaziny, pyrrolidiny, lower alkyl substituted pyrrolidiny, piperidiny and lower alkyl substituted piperidiny; R_7 is lower alkyl; and R_8 is selected from the group consisting of lower alkyl and hydrogen

and water soluble acid addition salts thereof to color said solution; and

(d) colorimetrically quantitating the iron content of said solution by means of said color.

6. The process of claim 5 wherein said compound is 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

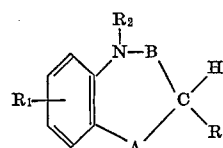
7. The process of claim 5 wherein said compound is 7-bromo-1,3-dihydro-1-[4-(4-methyl-1-piperaziny)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

8. The process of claim 5 wherein said iron is extracted with an acid deproteinizing agent.

9. The process of claim 8 wherein said deproteinizing agent is trichloroacetic acid.

10. A process for quantitating the hemoglobin content of whole fresh blood comprising:

- extracting the iron in said blood into an aqueous solution in the ferric state;
- treating the ferric ions in said aqueous solution with a reducing agent to reduce the ferric ions to ferrous ions;
- reacting the ferrous ions with a compound selected from the group consisting of compounds of the formula



wherein A is selected from the group consisting of



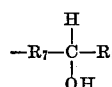
and



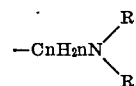
B is selected from



and $-CH_2-$; R_1 is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R_2 is selected from the group consisting of



hydrogen, lower alkyl and



n is an integer from 2 to 7; R_3 is selected from the group consisting of hydrogen, hydroxy, lower alkyl,

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lower alkoxy and lower alkanoyloxy; R_4 is 2-pyridyl; R_5 is selected from the group consisting of lower alkyl and hydrogen; R_6 is selected from the group consisting of lower alkyl, hydrogen,



and $\text{---C}\equiv\text{N}$; and R_5 and R_6 where taken together with their attached nitrogen atom form a radical selected from the group consisting of piperaziny, lower alkyl substituted piperaziny, pyrrolidiny, lower alkyl substituted pyrrolidiny, piperidiny and lower alkyl substituted piperidiny; R_7 is lower alkyl; and R_8 is selected from the group consisting of lower alkyl and hydrogen

and water soluble acid addition salts thereof to produce colored solutions;

(d) colorimetrically quantitating the iron present in the solution by means of said color; and

(e) calculating the hemoglobin content based upon the quantitative determination of the iron present in said solution.

11. The process of claim 10 wherein said compound is 7-bromo - 1,3 - dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

12. The process of claim 10 wherein said compound is 7-bromo-1,3-dihydro-1-[4-(4-methyl-1-piperaziny)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepine.

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13. The process of claim 10 wherein said compound is 7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

14. The process of claim 10 wherein said iron is extracted by the addition of an aqueous hypochlorite salt.

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MORRIS O. WOLK, Primary Examiner

E. A. KATZ, Assistant Examiner

U.S. Cl. X.R.

252—408; 260—239.3; 424—2

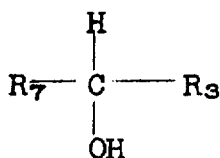
UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,506,404 Dated April 14, 1970

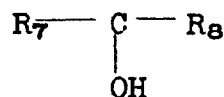
Inventor(s) Evans and Searcy

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

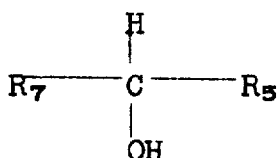
Column 8, lines 72-75



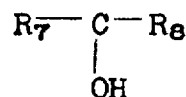
should be



Column 9 lines 67 - 70



should be



SIGNED AND
SEALED
JAN 5 1971

(SEAL)

Attest:

Edward M. Fletcher, Jr.

Attesting Officer

WILLIAM E. SCHUYLER, JR.
Commissioner of Patents